Vascular Endothelial Growth Factor Receptors in Human Mesangium in Vitro and in Glomerular Disease

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Abstract. Mesangial cell proliferation and growth factor overexpression are characteristic features of several glomerular diseases. Vascular endothelial growth factor (VEGF), a potent mitogen, is expressed in podocytes in the glomerulus, and VEGF receptors (flt-1, KDR, and neuropilin-1) are present on endothelial cells and other cell types. This study examined whether human mesangial cells (HMC) express VEGF receptors and the VEGF receptors and VEGF action on HMC have not been studied. We therefore investigated VEGF receptors and the effect of VEGF on HMC proliferation. All receptor types were detected in HMC in vitro by immunofluorescence and Western blotting. VEGF165 induced a dose-responsive increase in 3H-thymidine incorporation (25 ng/ml VEGF165: 2.3-fold increase; 50 ng/ml: 3.8-fold; 100 ng/ml: 4.8-fold; 200 ng/ml: 3.4-fold; P = 0.016) and in cell number (50 ng/ml VEGF165: 1.2-fold increase; 100 ng/ml: 1.6-fold; 200 ng/ml: 1.4-fold; P = 0.005), effects prevented by an anti-VEGF165 polyclonal neutralizing antibody (100 μg/ml). The proliferative effect was confirmed by a tetrazolium dye-based assay (100 ng/ml VEGF165: 1.4-fold increase). In ex vivo experiments, VEGF receptors in biopsy material from normal and diseased kidneys were detected by immunohistochemistry. No mesangial flt-1 receptor staining was seen in normal renal cortical tissue samples, and only weak mesangial KDR staining was detected. In contrast, mesangial flt-1 and KDR receptor staining were both clearly seen in biopsy samples from proliferative renal diseases. In conclusion, flt-1, KDR, and neuropilin-1 are present on cultured HMC, and VEGF165 induces HMC proliferation. In addition, the flt-1 and KDR receptors are expressed in the mesangium in mesangioproliferative disease.

Growth factors and cytokines are important in promoting mesangial cell proliferation, a characteristic feature of several different glomerular diseases (1,2). Vascular endothelial growth factor (VEGF) is a disulfide-linked homodimeric glycoprotein of 34 to 46 kD (3). Alternative splicing of the VEGF mRNA generates five isoforms (VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206), of which VEGF165 appears to be the most abundantly secreted (4). VEGF is induced by transforming growth factor-β1 (5) and platelet-derived growth factor via protein kinase C activation (6), and is an extremely powerful permeability factor and a potent mitogen for endothelial cells (4). It induces the production of factors such as plasminogen activator inhibitor-1 and collagenase, which are important in matrix remodeling (7). There are three known VEGF receptors, the class III receptor tyrosine kinases flt-1 (8), KDR (also known as flk-1 in the mouse) (9,10), and the recently identified neuropilin-1, which enhances binding of VEGF165 to KDR (11). Initially thought to act exclusively on endothelial cells, VEGF has recently been shown to bind to Balb/C, HeLa, and melanoma cells (12,13) and induce proliferation in retinal pigment epithelial cells (14,15). Furthermore, VEGF increases the mitogenic effect of fibroblast growth factor-2 on vascular smooth muscle cells via flt-1 (16), activates mitogen-activated protein kinases in cardiac myocytes (17), and has procoagulant and chemotactic actions on monocyte-macrophage cells (18), all cell types with similarities to human mesangial cells (HMC).

VEGF has been implicated in the induction of proteinuria in renal disease (19), and in vitro glomerular mesangial cells produce VEGF, which can be stimulated by fetal calf serum (FCS) (20), transforming growth factor-β1 (5), angiotensin II (21), and the application of mechanical stretch (22). In addition, the upregulation of VEGF in the mesangium of mesangial proliferative disease was recently described (23). To date, VEGF receptors and VEGF action on HMC have not been studied. We therefore investigated VEGF receptors and the effect of VEGF165 on HMC in vitro and examined the presence of VEGF receptors in the mesangium from human kidney biopsy material to determine whether VEGF165 plays a role in mesangial cell pathophysiology.

Materials and Methods

Materials

All materials were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. RPMI 1640 culture medium and FCS.
were obtained from Life Technologies (Paisley, United Kingdom). Rabbit polyclonal anti-human VEGF<sub>165</sub> and anti-Thy-1 antibodies were from Serotec (Oxford, United Kingdom). Anti-flt-1 receptor antibody, anti-KDR antibody, flt-1 blocking peptide, and control antibodies were obtained from Santa Cruz Biotechnology (Middlesex, United Kingdom). Anti-neuropilin-1 and recombinant human VEGF<sub>165</sub> were from R&amp;D Systems (Oxon, United Kingdom). Antibodies against cytokeratin, factor VIII, common leukocyte antigens, tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-rabbit swine serum, and reagents for immunoperoxidase staining were obtained from DAKO (High Wycombe, United Kingdom). All materials for Western analysis were purchased from Amersham Life Science (United Kingdom) and Bio-Rad (United Kingdom).

**Cell Culture**

HMC were isolated as described previously (24). Briefly, normal renal cortex for the in vitro studies was obtained from three kidneys. Two were obtained from the opposite tumor-free pole of nephrectomy specimens, removed for localized hypernephromas, and one was obtained from a donor nephrectomy found to be unsuitable for transplantation on the basis of an abnormal vascular supply. The cortical tissue was analyzed histologically to confirm the absence of tumor cells or other renal pathology. Intact glomeruli were collected from cortical homogenates by serial sieving. The isolated glomeruli were digested with collagenase (type IV, 750 U/ml) and then seeded in culture flasks. After the outgrowth of HMC, the glomeruli were washed and the cells were cultured in RPMI 1640, supplemented with insulin-transferrin-selenium, 20% FCS, 7 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO<sub>2</sub> incubator at 37°C. HMC were passaged using 0.25% trypsin and 0.5% ethylenediaminetetraacetic acid. The cells were stellate or fusiform in appearance, grew in multilayers, formed a monolayer derived from explanted tumors, and retained a fibroblastoid appearance. Normal, unstimulated cultures of HMC grew in multilayers, formed a monolayer derived from explanted tumors, and retained a fibroblastoid appearance.

**VEGF Receptor Analysis**

**Immunofluorescence.** HMC were grown on glass slides and fixed in 100% methanol for 4 min at −20°C. They were then incubated with 10% normal swine serum for 20 min, followed by either a rabbit polyclonal anti-human flt-1 antibody (10 μg/ml in phosphate-buffered saline-bovine serum albumin), a rabbit polyclonal anti-KDR antibody (10 μg/ml), or a goat polyclonal anti-neuropilin-1 antibody (10 μg/ml) for 60 min at 37°C. After rinsing, the cells were incubated with a TRITC-swine anti-rabbit antibody conjugate (TRITC-rabbit anti-goat antibody conjugate for neuropilin-1) for 60 min. The cells were then incubated for 1 h with either a polyclonal anti-human flt-1 antibody, a polyclonal anti-human KDR antibody, or a polyclonal anti-human neuropilin antibody (1 μg/ml in TBS-T and 0.5% nonfat milk), extensively washed in TBS-T then incubated with horseradish peroxidase-linked antibodies (1:1500 dilution). Signal was performed by enhanced chemiluminescence. Negative controls consisted of: (1) omission of the primary antibody, (2) an flt-1 blocking peptide (100 μg/ml), and (3) goat nonimmune IgG at the same concentration as the primary antibody.

**Cell Proliferation**

HMC were grown to confluence, passaged, and seeded at 35,000 cells per well. After 48 h of serum deprivation, the cells were treated with recombinant human VEGF<sub>165</sub> for 24 h. Neutralization experiments were performed in the presence or absence of a rabbit anti-human VEGF<sub>165</sub> polyclonal neutralizing antibody (100 μg/ml).

**3H-Thymidine Incorporation.** 3H-Thymidine incorporation was measured in TCA-precipitable material (26). 3H-Thymidine (1 μCi/ml) was added to experimental media for 24 h. The cells were washed and incubated three times in ice-cold 0.6 M TCA at 4°C for 20 min each time. The cells were then solubilized by 0.2N perchloric acid. Incorporated radioactivity was measured using a liquid scintillation counter (Packard Tri-Carb Liquid Scintillation Analyzer model 1900 CA).

**Cell Number.** The cell number was determined after cell separation using trypsin-ethylene glycol tetraacetic acid by Coulter counter analysis, using an aperture size of 13 to 49 μM (model ZI; Coulter Electronics, Hialeah, FL).

Cell proliferation was also assessed using a tetrazolium dye assay based on the conversion of 3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan by living cells (27). The insoluble formazan was solubilized with 100% ethanol and measured spectrophotometrically at 560 nm.

**Ex Vivo Studies: Immunohistochemistry**

**Subjects.** Normal cortical renal tissue was obtained from five localized tumor nephrectomy samples for the ex vivo studies (patients age range, 50 to 75 yr) and processed in an identical manner to biopsy specimens from renal diseases. After retrieval, the samples were immersed in liquid nitrogen for 1 h and then stored at −70°C until analysis. The cortical samples were taken from the tumor-free pole, and normal pathology was confirmed by light microscopy. Renal cortical samples were also analyzed from biopsy samples of five patients (age range, 30 to 75) with proliferative renal diseases. Three patients had IgA nephropathy and presented with microscopic hematuria and mild renal impairment, with a serum creatinine level <150 μmol. One patient had diffuse mesangial proliferative glomerulonephritis with mesangial IgM and presented with nephrotic syndrome and a creatinine clearance of 29 ml/min,
and another had acute diffuse mesangial proliferative glomerulonephritis with immune-complex deposits and presented with microscopic hematuria and proteinuria (1.1 g/24 h).

Immunoperoxidase staining was carried out on 5-μm frozen tissue sections fixed in acetone at −4°C for 10 min. Tissue sections were washed in TBS-T, pH 7.4, then blocked for 30 min with 5% goat serum in TBS-T for flt-1 and 10% goat serum in TBS-T for KDR. Primary antibodies against anti-flt1 (2 μg/ml) and KDR (5 μg/ml) diluted in TBS-T with 5 to 10% goat serum were incubated for 1 h at room temperature. The samples were then incubated with a peroxidase-labeled polymer conjugated to goat anti-rabbit IgG (Envision System; Dako) for 30 min. Intermediate washes were done with TTBS. For visualization, the sections were developed with 3,3′-diaminobenzidine to produce a brown color, counterstained with hematoxylin, and finally mounted in DePeX mounting medium. Control experiments were performed in parallel. The negative control was by omission of the first antibody and the positive control was factor VIII at a dilution of 1:300.

Statistical Analyses
Comparisons among groups were analyzed by ANOVA and between experiments by the Student-Newman-Keuls test using Statistical Package for the Social Sciences software. Differences were considered significant at P < 0.05. All data points were determined in duplicate. Data are reported as means ± SD.

Results
HMC Express KDR, flt-1, and Neuropilin-1 Receptors in Vitro

Immunofluorescence. Focal areas of intensity were seen with anti-KDR (Figure 1A), anti-flt-1 (Figure 1B), and anti-neuropilin-1 (Figure 1C) antibodies. These were distributed over the cell surface in a pattern suggestive of a membrane-bound receptor, with cytoplasmic staining as has been described previously for VEGF receptors (28). The pattern and intensity of the staining to both receptors were similar to that seen in HUVEC used as a positive control (not shown).

Preincubation with VEGF165 (100 ng/ml) for 30 min resulted in virtually no specific staining, as did omission of the primary antibody (Figure 1D). Similarly, the addition of a specific anti-flt-1 antibody blocking peptide (100 μg/ml) also prevented staining of the flt-1 receptor, confirming the specificity of the anti-flt-1 antibody. There was no specific staining seen using nonimmune IgG as the primary antibody.

Western Blotting. Total protein lysates, from HMC serum and insulin deprived for 48 h, were analyzed. Using specific antibodies, all three VEGF receptors—KDR with a molecular weight of 180 kD, flt-1 with a molecular weight of 180 kD, and neuropilin-1, molecular weight 130 kD (9,29,30)—were detected in HMC (Figure 2). These findings were consistent with the known molecular weights of these receptors and were identical to those seen using HUVEC as a positive control. The specificity of the band was confirmed by the absence of specific signal in the negative controls.

VEGF Induces HMC Proliferation

3H-Thymidine Incorporation. In time–response experiments, serum- and insulin-deprived cells were exposed to VEGF165 (100 ng/ml) for 6, 12, and 24 h.

Figure 1. Immunofluorescence staining of vascular endothelial growth factor (VEGF) receptors on human mesangial cells (HMC). HMC were grown on glass slides, fixed, and incubated with specific antibodies for 60 min at 37°C. The cells were examined using a fluorescence microscope (Olympus BX60 at ×40 magnification) and photographed using an Olympus SC35 Type 12 with Provia 1600 daylight film (FujiChrome). The cells were incubated with rabbit polyclonal anti-human flt-1 antibody (A), a rabbit polyclonal anti-KDR receptor (B), and a goat polyclonal anti-neuropilin-1 antibody (C). An example of a negative control is also shown (D).
There was a 1.6-fold increase in $^3$H-thymidine incorporation by 6 h, rising to 3.3-fold at 12 h and 4.8-fold at 24 h. The 24-h time point was thus chosen for further experiments.

In dose–response experiments, a dose-dependent increase in HMC $^3$H-thymidine incorporation was seen, with a peak 4.8-fold increase at 100 ng/ml (mean $\pm$ SD fold increases of 2.3 $\pm$ 0.99, 3.8 $\pm$ 1.69, 4.8 $\pm$ 2.3, and 3.4 $\pm$ 2.5 at concentrations of 25, 50, 100, and 200 ng/ml, respectively) ($P = 0.016; n = 5$) (Figure 3A).

**Cell Number.** To establish whether an increase in $^3$H-thymidine incorporation was paralleled by an increase in cell number, serum- and insulin-deprived HMC were seeded in equal number and VEGF$_{165}$ was added at doses of 50, 100, and 200 ng/ml for 24 h. Cell number was determined by Coulter analysis after 24 h.

The final cell number was VEGF$_{165}$ concentration-dependent, with a peak 1.6-fold increase at a concentration of 100 ng/ml (mean $\pm$ SD fold increases of 1.2 $\pm$ 0.27, 1.6 $\pm$ 0.69, and 1.4 $\pm$ 0.33 at 50, 100, and 200 ng/ml, respectively) ($P = 0.005; n = 6$) (Figure 3B).

Similar results were also obtained in a subset of experiments, using a tetrazolium dye-based assay of cell proliferation that estimates numbers of viable cells. VEGF$_{165}$ (100 ng/ml) induced a consistent 1.4-fold increase in HMC proliferation after 24 h ($n = 3$).

**Neutralization Experiments.** The addition of a polyclonal VEGF-neutralizing antibody prevented VEGF$_{165}$-induced proliferation as assessed by both $^3$H-thymidine incorporation (Figure 3C) and cell number (Figure 3D).

**Ex Vivo Studies: Immunohistochemistry**

To exclude that the VEGF receptor expression was not solely related to *in vitro* culture conditions and given the effect

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**Figure 2.** VEGF receptors are expressed in HMC *in vitro*. Cell lysates from human umbilical vein endothelial cells (A) and HMC (B) were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Bands of molecular weight corresponding to the VEGF receptors (KDR, neuropilin-1, and flt-1) were detected by immunoblotting using specific antibodies.

**Figure 3.** VEGF effect on HMC proliferation. Serum- and insulin-deprived HMC were exposed to VEGF$_{165}$ (25, 50, 100, and 200 ng/ml) for 24 h, and $^3$H-thymidine incorporation (A) and cell number (B) were determined as described in Materials and Methods. Serum- and insulin-deprived HMC were exposed to VEGF$_{165}$ (100 ng/ml) for 24 h in the presence and in the absence of a VEGF-neutralizing antibody (100 $\mu$g/ml), and $^3$H-thymidine incorporation (C) and cell number (D) were determined. *$P = 0.016$; **$P = 0.005$. 

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of VEGF on mesangial cell proliferation, we examined expression of VEGF flt-1 and KDR, the principal VEGF receptors, in normal cortical tissue and in proliferative glomerulonephritis. In normal cortical tissue from nephrectomy specimens, there was strong positive staining for both flt-1 and KDR in the glomerular capillary walls. In the mesangium, there was no specific flt-1 staining, but there was weak KDR immunoreactivity. In contrast, in proliferative glomerulonephritis samples, in addition to the glomerular capillary staining, there was strong specific flt-1 and KDR in the mesangium (Figure 4). In both normal and disease tissue, some staining of proximal tubular cells was also seen, as has been described previously (31). No staining was seen in negative controls.

Discussion

This work describes the presence of flt-1, KDR, and neuropilin-1 VEGF receptors on HMC and reports that VEGF induces HMC proliferation. We also identified the principal two VEGF receptors, flt-1 and KDR, in the mesangium of pathologic tissue. VEGF is a potent mitogen for endothelial cells, and originally these were thought to be the only target for its actions. Our findings expand recent evidence that VEGF can act on a variety of cell types. In the kidney, VEGF is strongly expressed in glomerular epithelial cells (32), although its role in the glomerulus is not yet fully understood. KDR and flt-1 were identified as VEGF receptors by affinity cross-linking and competition binding assay (33). Recently, neuropilin-1, a previously characterized neuronal cell surface protein important in axon growth in the developing embryo, was identified as a VEGF<sub>165</sub>-specific receptor on endothelial cells (11). It appears to be responsible for augmenting VEGF<sub>165</sub> binding to KDR and is widely expressed in human tissues including the kidney (11).

In the present study, immunofluorescence revealed in HMC in vitro flt-1, KDR, and neuropilin-1 with a staining pattern similar to that seen in human umbilical vein endothelial cells used as a positive control. VEGF receptors colocalize with caveolin-1 in plasma membrane caveolae (28), which explains why the staining pattern was that of a membrane-bound receptor coupled with cytoplasmic staining. There was no nuclear staining as has been described previously with VEGF receptors (28). Preincubation with recombinant human VEGF prevented detection of the receptors, confirming the specificity of the staining for VEGF receptors. This was further confirmed by the addition of soluble flt-1 as a blocking peptide, which prevented staining detected using the anti flt-1 antibody. The receptors were further identified in HMC lysates by Western blotting.

The addition of VEGF to cultured HMC induced mesangial cell proliferation. VEGF at 25 ng/ml stimulated a 2.3-fold increase in HMC ³H-thymidine incorporation, with a peak 4.8-fold increase at a dose of 100 ng/ml. At this dose, there was also a significant 1.6-fold increase in cell number. Both ³H-thymidine incorporation and the increase in cell number were prevented by the addition of specific VEGF-neutralizing antibodies, indicating that these are specific effects of VEGF. Differences in magnitude of the effect as measured by the different methodologies probably reflect the fact that ³H-thymidine incorporation is not a direct estimate of cell number. Indeed, the two methods more directly estimating cell number, namely the Coulter counter method and the tetrazolium dye assay, gave very similar results. The maximum effect was seen at VEGF<sub>165</sub> concentrations of approximately 100 ng/ml, higher than those that induce endothelial cell proliferation and collagenase production (34,35), but comparable to those that induce

Figure 4. VEGF receptor expression in mesangial proliferative glomerulonephritis. Immunohistology for flt-1 and KDR was performed by immunoperoxidase and counterstained with hematoxylin. In normal tissue, staining was exclusively seen in the glomerular capillary wall (Panel A, flt-1). In pathologic tissue, flt-1 (Panel B, acute proliferative glomerulonephritis) and KDR (Panel C, IgA nephropathy) were seen in the mesangium (large arrow) and in the glomerular capillary wall (small arrow). KDR staining was also detectable in the extraglomerular capillaries (arrowhead). For technical details, see Materials and Methods. Magnification, ×40.
monocyte migration (18) and pericyte proliferation (36). Notably, HMC in culture produce VEGF in concentrations of this order (20), suggesting that these concentrations may be pathophysiologically relevant. In our study, there was no further increase in either ³H-thymidine incorporation or cell number at doses of 200 ng/ml, a feature also seen in other cell types (34,35), which may indicate saturation at the receptor or postreceptor level.

The present study did not determine which receptor is important in mediating VEGF-induced mesangial cell proliferation. The mitogenic and gross morphologic effects of VEGF on endothelial cells (37) appear to be mediated via KDR, whereas flt-1 appears important in angiogenesis (38) and in VEGF-induced monocyte migration (39). In vivo, both flt-1 and KDR appear to be upregulated by hypoxia, whereas in vitro hypoxia leads to strong transcriptional activity of the flt-1 promoter with no change, or even a downregulation, in KDR transcriptional activity (38). Hypoxia is an unlikely explanation for the finding of VEGF receptors in the present study, because the HMC were cultured at normal oxygen concentrations, well above those used in the studies of hypoxia.

Two of the kidney specimens used for mesangial cell culture were obtained from tumor nephrectomies. Although we used only tissue distant from a localized tumor, it was possible that this could influence our findings because VEGF and the VEGF receptors are overexpressed in renal cell carcinomas (40). To exclude this possibility, we obtained a normal cadaveric kidney unsuitable for transplantation. The results in cells derived from this kidney were superimposable on those of cells from tumor nephrectomies.

We confirmed our in vitro findings in human biopsy material. We studied “normal” kidneys and, as an example of a disease state, we chose mesangiotrophic glomerulonephritis because we had shown in vitro that VEGF stimulated mesangial cell proliferation. In the normal kidney, staining for VEGF receptors was detected in the capillary wall, but no flt-1 staining was seen in the mesangium and there was only weak KDR staining. This is consistent with the previously reported physiologic location of these receptors (32). In contrast, both flt-1 and KDR are both clearly present in the mesangium in biopsies of patients with mesangial proliferative glomerulonephritis. This is in accord with the observed upregulation of the KDR receptor seen in animal models of mesangiotrophic disease (41) and nephrosis (42), and human studies of early mesangiotrophic disease. Importantly, in both animal models of renal disease and in early human mesangiotrophic glomerulonephritis, upregulation of mesangial VEGF has also been described (23,41).

Mesangial cell proliferation is a common response of the glomerulus to several diverse injuries in both human and experimental glomerular disease, and several growth factors are implicated in this process. There are several potential sources of glomerular VEGF. VEGF can be released by both resident glomerular cells and by infiltrating T lymphocytes (43) and monocytes (5). Of glomerular cells, epithelial (32), endothelial (44), and mesangial (5) cells produce VEGF in vitro. VEGF is upregulated by several growth factors, inflammatory cytokines (45), and vasoactive agents in the kidney (46). Similarly, known inducers of the VEGF receptor include serum, platelet-derived growth factor, and hypoxia (47). Thus, a series of stimuli involved in glomerular injury can induce both VEGF and its receptor. Our results raise the possibility that VEGF/VEGF receptor systems are upregulated in glomerular disease and may play an important role in mesangial cell pathophysiology.

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